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Elevated mitochondrial biogenesis in skeletal muscle is associated with testosterone-induced body weight loss in male mice

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ABSTRACT

Androgen reduces fat mass, although the underlying mechanisms are unknown. Here, we examined the effect of testosterone on heat production and mitochondrial biogenesis. Testosterone-treated mice exhibited elevated heat production. Treatment with testosterone increased the expression level of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), ATP5B and Cox4 in skeletal muscle, but not that in brown adipose tissue and liver. mRNA levels of genes involved in mitochondrial biogenesis were elevated in skeletal muscle isolated from testosterone-treated male mice, but were down-regulated in androgen receptor deficient mice. These results demonstrated that the testosterone-induced increase in energy expenditure is derived from elevated mitochondrial biogenesis in skeletal muscle.

1. Introduction

Testosterone deficiency leads to increased fat mass and insulin resistance [1]. Epidemiological studies demonstrate that low testosterone level is associated with type 2 diabetes and metabolic syndrome [2,3]. A double-blind placebo-controlled study reveals that testosterone replacement therapy reduces HOMA index, fasting glucose, HbA1c and waist circumference in type 2 diabetic patients with hypogonadism [4]. Our previous study demonstrated that treatment with dehydroepiandrosterone (DHEA) and testosterone equally reduces adiposity in rats [5]. Both DHEA and testosterone equally suppress 3T3-L1 preadipocyte proliferation and expression of PPARγ in 3T3-L1 adipocytes. In this study, we found that these effects of DHEA are mediated via the androgen receptor (AR). However, the mechanism of weight reduction is unclear. Since neither DHEA nor testosterone suppresses food consumption, we hypothesized that they may increase energy expenditure. This prompted us to examine the effect of treatment with testosterone on oxygen consumption. As we found that an increase in serum testosterone level enhances heat production without increasing locomotor activity, we investigated the effect of testosterone on mitochondrial biogenesis and the expression of its key regulator, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α).

2. Materials and methods

2.1. Animals

C57/Black male mice at 8 weeks of age were fed CE2 powder with (testosterone group) or without (control group) 0.4% testosterone ad libitum for 4 weeks. They were housed in a specific pathogen-free facility with a 12-h light/12-h dark cycle. Androgen receptor deficient mice (ARKO) were established with Cre-lox system as described previously [6,7]. They were fed CE2 ad libitum. After the treatment with testosterone, the animals were decapitated to collect epididymal fat (WAT), brown adipose tissue (BAT), liver and gastrocnemius muscle. In addition, these organs were harvested at 8 and 25 weeks of age in ARKO.

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All procedures for animal care were carried out in accordance with protocols approved by the University of Gifu's Institutional Animal Care Committee.

2.2. Measurement of oxygen consumption and locomotor activity

O_2 consumption, CO_2 production and locomotor activity were measured by indirect calorimetry as described previously [8].

2.3. Cell culture

C_2C_{12} myoblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. When cells reached 90% confluence, the medium was exchanged for DMEM containing 4% horse serum (differentiation medium). After the incubation with differentiation medium for 7 days, cells were morphologically determined to differentiate C_2C_{12} myotubes. C_2C_{12} myotubes were treated with various concentrations (0, 1, 10 and 100 nM) of testosterone for 48 h.

2.4. Assay for serum testosterone concentration

Serum testosterone concentration was measured with DPC Total Testosterone Kit (Diagnostic Products Corporation).

2.5. Real time PCR

Total RNA from gastrocnemius muscle isolated from mice treated with testosterone and ARKO, and C_2C_{12} myotubes were extracted using TRIzol, and purified as described previously [9]. Reverse transcription was performed using a PrimeScript Reverse Transcriptase (TAKARA) genes were established using SYBR Premix Ex Taq Kit (TAKARA) according to the manufacturer's instructions. 20 μl of the reaction solution consisted of 2 μl of the template, 10 μl of SYBR Premix Ex Taq, 0.4 μl of 10 μM of each primer and 0.4 μl of ROX Reference Dye. PCR amplification was performed as follows: predenaturation for 1 cycle at 95 °C for 30 s, and 40 cycles at 95 °C for 5 s, 60–62 °C for 30 s using a Thermal Cycler Dice (TAKARA, Ohtsu, Japan). Expression levels, calculated as copy numbers in each sample, were normalized to the expression level of GAPDH. Oligonucleotide primers were designed, based on sequences from the GeneBank database (Supplemental Table).

2.6. Quantification of mitochondrial DNA (mitDNA)

Total DNA containing nuclear DNA and mitDNA was extracted using ZymoBead™ Genomic DNA kit (Zymo Research Corp, Irvine, CA). Real time PCR was performed as described above to quantify mitDNA (16S rRNA: CCGCAAGGGAAAGATGAAAGAC (f) and TCGTT

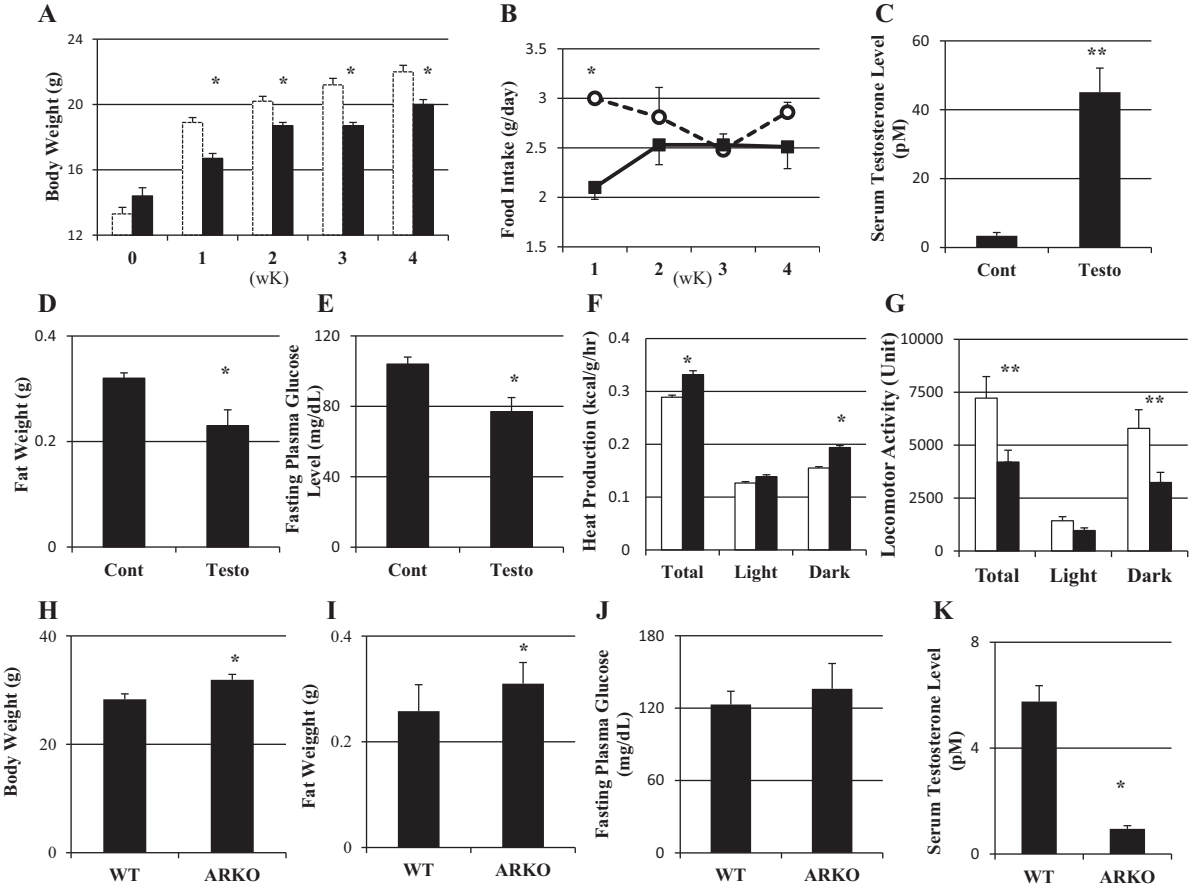

Fig. 1. Body weight, fat weight, plasma glucose level in testosterone-treated mice and ARKO. (A) C57/black mice were treated with food containing 0.4% testosterone for 4 weeks. Body weight in control (white) and treated (black) mice ($n = 16$, *: $P < 0.05$ control vs treated). (B) Food consumption in control (open circle-dotted line) and treated (closed square-solid line) mice ($n = 16$, *: $P < 0.05$ control vs treated). (C) Serum testosterone level in control and treated mice ($n = 8$, **: $P < 0.01$ control vs treated). (D) Epididymal fat weight in control and treated mice ($n = 16$, *: $P < 0.05$ control vs treated). (E) Fasting plasma level in control and treated mice ($n = 8$, *: $P < 0.05$ control vs treated). (F) Calculated heat production in control (white) and treated (black) mice during light phase (12 h), dark phase (12 h) and total (24 h) ($n = 7$, *: $P < 0.05$ control vs treated). (G) Locomotor activity in control (white) and treated (black) mice during light phase (12 h), dark phase (12 h) and total (24 h) ($n = 7$, *: $P < 0.05$ control vs treated). (H) Body weight in wild type and ARKO mice at 24 weeks of age ($n = 3$, *: $P < 0.05$ wild type vs ARKO). (I) Epididymal fat weight in wild type and ARKO mice at 24 weeks of age ($n = 3$, *: $P < 0.05$ wild type vs ARKO). (J) Plasma glucose levels in wild type and ARKO ($n = 3$, *: $P < 0.05$ wild type vs ARKO). (K) Serum testosterone levels in wild type and ARKO ($n = 3$, *: $P < 0.05$ wild type vs ARKO).

TGGTTTCGGGGTTTC (r)) and nuclear DNA (cyclophilin A: TTCCTCC TTTCACAGAATTATTCCA (f) and CCGCCAGTGCCATTATGG (r)) simultaneously in 10–10000 times diluted total DNA. The copy number of mitDNA was normalized with nuclear DNA. We assayed the amount of mitDNA in skeletal muscle isolated from C57/Black male mice treated with or without testosterone for 4 weeks as described above.

2.7. Western blotting

Gastrocnemius muscle, WAT, liver and BAT were homogenized in RIPA buffer (Sigma Aldrich, St. Louis MO). Cell lysates were mixed with Laemmli sample buffer and boiled for 3 min. Equal amounts of cell lysate were subjected to SDS–PAGE, and transferred onto nitrocellulose paper. The paper was blocked with 5% skim milk TBS, and incubated with anti-PGC1 antibody (Santa Cruz Biotech, Santa Cruz, CA), anti-ATP5B antibody (Aviva Systems Biology, San Diego, CA), anti-Cox4 antibody (Gene Tex, Irvine, CA) or anti-cytochrome *C* antibody (Gene Tex, Irvine, CA). Protein bands were visualized with an ECL system.

2.8. Immunohistochemical staining

Mitochondria staining of paraffin sections of each tissue was carried out using anti-ATP5B antibody (Aviva Systems Biology, San Diego, CA, USA), and Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

2.9. Statistics

Statistical comparisons were performed with Student *t*-test (Figs. 1C–K and 3B–F) one-factor ANOVA (Figs. 2B–F and 1A–H). All statistical tests were two-tailed. Data are given as mean ± S.E.M. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of treatment with testosterone on body weight, food consumption, and plasma glucose in male C57Black mice and ARKO

Treatment food containing 0.4% testosterone suppressed the body weight gain, despite no significant decrease in food consumption being observed except during the first week (Fig. 1A and B). Serum testosterone level was elevated up to approximately 12-fold in the testosterone group (Fig. 1C), which was accompanied with decreases in epididymal fat weight and fasting plasma glucose level (Fig. 1D and E). These results indicated that testosterone-induced weight loss is not fully explained by decreased food consumption alone.

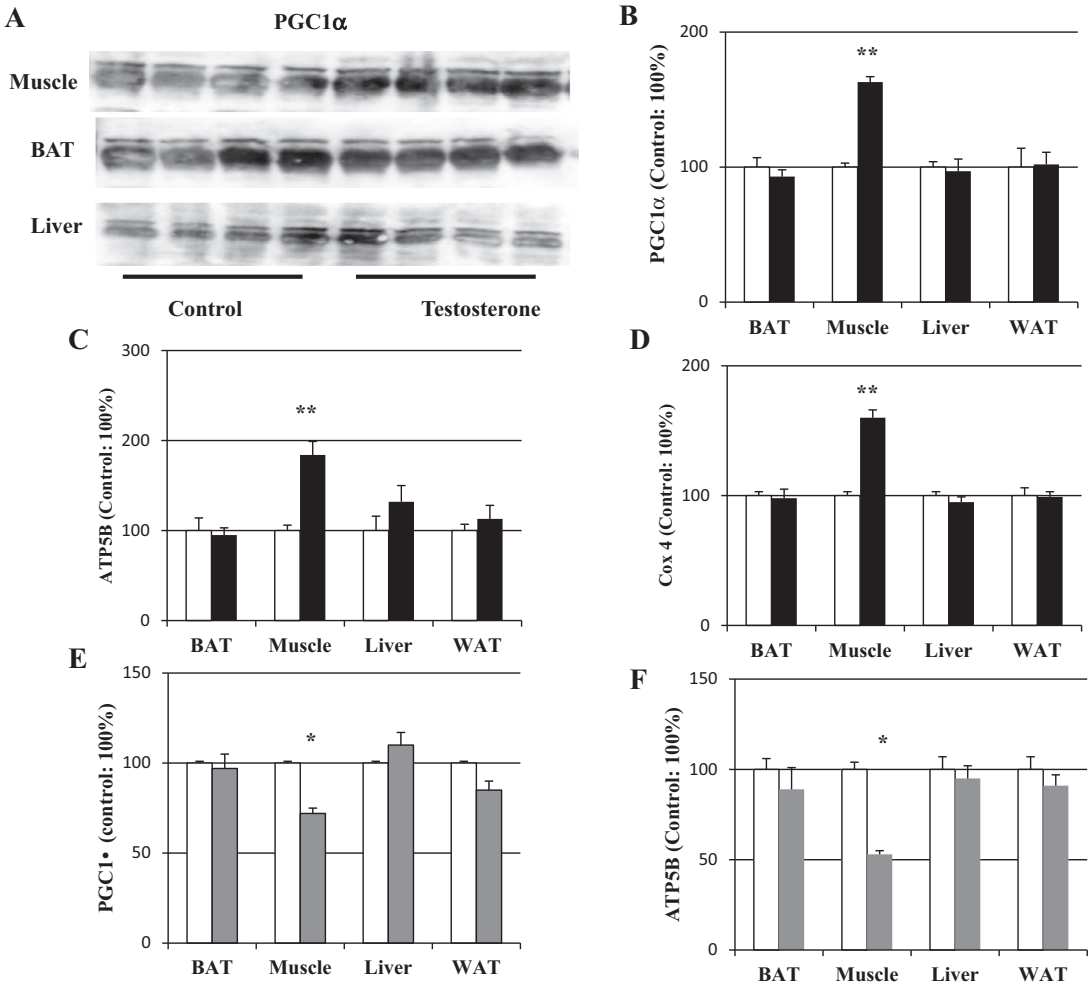

Fig. 2. Expression levels of PGC1α and mitochondrial protein in various organs isolated from testosterone-treated mice and ARKO. (A) Western blot showing the PGC 1α expression levels in muscle, BAT and liver isolated from control and treated mice. (B–D) Quantified value of PGC1α (B), ATP5B (C) and Cox4 (D) protein levels in control (white) and testosterone-treated (black) mice ($n = 8$, **: $P < 0.01$, control vs treated mice). (E, F) Quantified value of PGC1α (E) and ATP5B (F) protein levels in wild type (white) and ARKO (gray) mice ($n = 3$, *: $P < 0.05$, wild type vs ARKO).

Heat production during 24 h and the light (resting) phase was elevated up to 114% and 124%, respectively (Fig. 1F), whereas locomotor activity in 24 h and the dark phase was suppressed in the testosterone-treated mice (Fig. 1G). Respiratory quotient was not influenced by treatment with testosterone.

Body weight and epididymal fat weight were increased in ARKO at 25 weeks of age compared with their wild type littermates (Fig. 1H and I), with no difference however observed in fasting plasma glucose (Fig. 1J). Serum testosterone level was decreased in ARKO at 25 weeks compared with wild type (Fig. 1K). No differences in body weight, fat weight or fasting plasma glucose level were observed between ARKO and wild type at 8 weeks of age, as described previously [6] (data not shown).

3.2. Effects of treatment with testosterone on mitochondrial biogenesis

Next, we evaluated the effect of testosterone on the expression levels of PGC1α and mitochondrial biogenesis in skeletal muscle (gastrocnemius muscle), liver, BAT and WAT. We observed elevated protein levels of PGC1α only in skeletal muscle isolated from testosterone-treated mice (Fig. 2A and B). In addition, treatment with testosterone increased the expression of protein levels of ATP5B and Cox4 in skeletal muscle (Fig. 2C and D). Conversely, expression levels of PGC1α and ATP5B protein were suppressed in skeletal muscle isolated from ARKO (Fig. 2E and F). The expression levels of PGC1α, Cox4 and ATP5B tended to decrease in skeletal muscle from ARKO, but not significantly so (data not shown). These results clearly imply that androgen increases expression of PGC1α and subsequent mitochondrial biogenesis exclusively in skeletal muscle.

Immunohistochemical study revealed that treatment with testosterone up-regulated the expression of mitochondrial Cox4 in skeletal muscle but not BAT (Fig. 3A). To confirm that mitochondrial biogenesis is elevated by treatment with testosterone in skeletal muscle, mRNA expression of genes involved in mitochondrial biogenesis including PGC1α, NRF-1, NRF-2 and Tfam was evaluated. As shown in Fig. 3B, mRNA levels of these genes were increased in testosterone-treated mice. Moreover, mitDNA (Fig. 3C) was elevated by treatment with testosterone. PGC1α converts muscle fibers from type II (fast twitch with abundant troponin T3 and troponin C2) to type I (slow twitch with abundant myoglobin and toponin I1) [10–12]. The expression levels of myoglobin and troponin I were increased in the testosterone-treated mice (Fig. 3D), which suggested that conversion of muscle fibers might be induced by testosterone. On the other hand, mRNA levels of these genes were reduced in skeletal muscle of ARKO (Fig. 3E). Furthermore, the ratio of myoglobin/troponin I was decreased in ARKO (Fig. 3F). These data indicated that treatment with testosterone increases the expression level of PGC1α, which leads to elevated mitochondrial biogenesis in skeletal muscle.

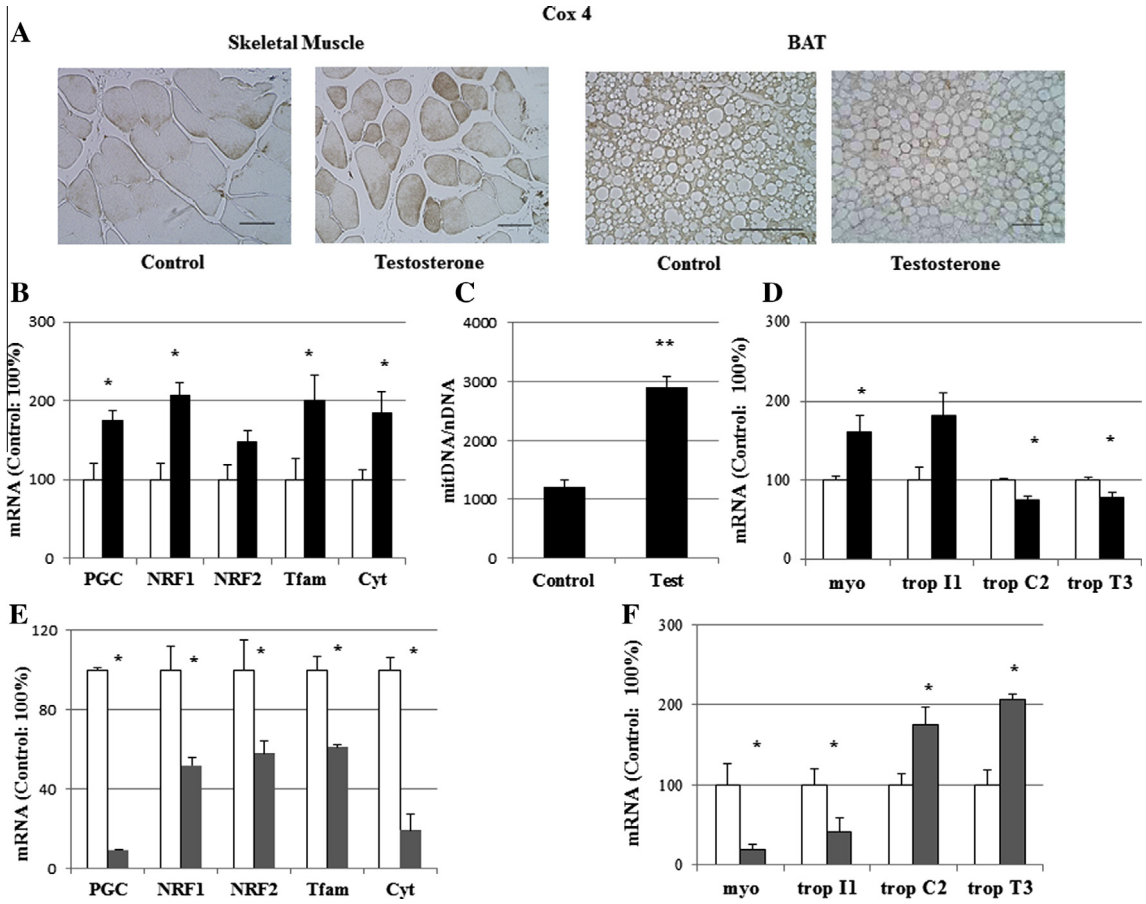

Fig. 3. Expression of genes involved in mitochondrial biogenesis in skeletal muscle. (A) Immunohistochemical study exhibiting the expression of Cox4 in skeletal muscle and BAT isolated from control and testosterone-treated mice. (B) Expression of mRNA in skeletal muscle isolated from control (white) and testosterone-treated (black) mice ($n = 6$, *: $P < 0.05$ control vs treated mice). (C) Amount of mitochondrial DNA (16S rRNA) adjusted by nuclear DNA (cyclophilin A) in skeletal muscle isolated from control and testosterone-treated mice ($n = 5$, *: $P < 0.05$ control vs treated mice). (D) Expression of myoglobin (Myo: type I muscle fiber) and troponin I1 (Trop I1: type I muscle fiber), troponin C2 (Trop C2: type II muscle fiber) and troponin T3 (Trop T3: type II muscle fiber) in skeletal muscle isolated from control (white) and testosterone-treated (black) mice ($n = 6$, *: $P < 0.05$ control vs treated mice). (E) Expression of mRNA in skeletal muscle isolated from wild type (white) and ARKO (gray) mice ($n = 3$ *: $P < 0.05$, **: $P < 0.01$, wild type vs ARKO). (F) Expression of myoglobin, troponin I1, troponin C2 and troponin T3 in skeletal muscle isolated from wild type (white) and ARKO (gray) mice ($n = 3$ *: $P < 0.05$, **: $P < 0.01$, wild type vs ARKO).

3.3. Effect of treatment with testosterone on mitochondrial biogenesis in C_2C_{12} myotubes

We examined the effect of treatment with various concentrations of testosterone for 48 h on mitochondrial biogenesis in C_2C_{12} myotubes. 10 nM testosterone significantly increased mRNA levels of genes related to mitochondrial biogenesis including PGC1α, NRF1, NRF2 and Tfam, as well as mitochondrial gene, cytochrome *C* (Fig. 4A–E). Testosterone-induced up-regulation of PGC1α and cytochorome *C* was abolished by the androgen receptor antagonist, flutamide, but not the estrogen receptor antagonist, fulvestrant (Fig. 4F and G). These results indicate that testosterone at physiological concentrations is effective in promoting mitochondrial biogenesis. In addition, the androgen receptor antagonist, flutamide, completely abolished the testosterone-induced elevations of PGC1α and cytochrome *C* mRNA. As estrogen receptor antagonist, fulvestrant, failed to influence the testosterone-induced mitochondrial biogenesis, these effects of testosterone must be mediated via the androgen receptor.

4. Discussion

Various clinical evidence has demonstrated that hypogonadism in men is frequently associated with type 2 diabetes [1]. Recent investigations have shown that this is common in non-Western countries [13,14]. Moreover, hypogonadotropic hypogonadism occurs commonly in type 2 diabetes [15]. Since testosterone is easily converted to estradiol, a serum low testosterone level is not equal to low androgen receptor activity. A study on ARKO demonstrated clearly that late onset obesity is observed in these mice [6], which implies that androgen signaling is required to prevent obesity in men.

Here, we investigated the mechanism of testosterone-induced weight reduction. Our previous study demonstrated that treatment with both DHEA and testosterone reduced the expression levels of PPARγ, aP2 and lipoprotein lipase in 3T3-L1 adipocytes [5] and adipose tissue [16]. Suppression of PPARγ activity promotes leanness in PPARγ-deficient mice [17] and Pro12Ala polymorphism in humans [18]. However, these hormones do not influence the gene expression levels involved in triglyceride synthesis and lipolysis. Since obesity is a state of pathological energy accumulation, weight reduction should be explained by a decrease in energy intake and/or increase in energy expenditure. The present results indicated that testosterone administration increases energy production without significant suppression of food intake. This result is consistent with the fact that oxygen consumption is decreased without an increase in food consumption in ARKO [6,19]. Decreased locomotor activity in testosterone treated mice was unexpectedly shown, because lowered spontaneous activity was observed in ARKO [19]. These results suggest that locomotor

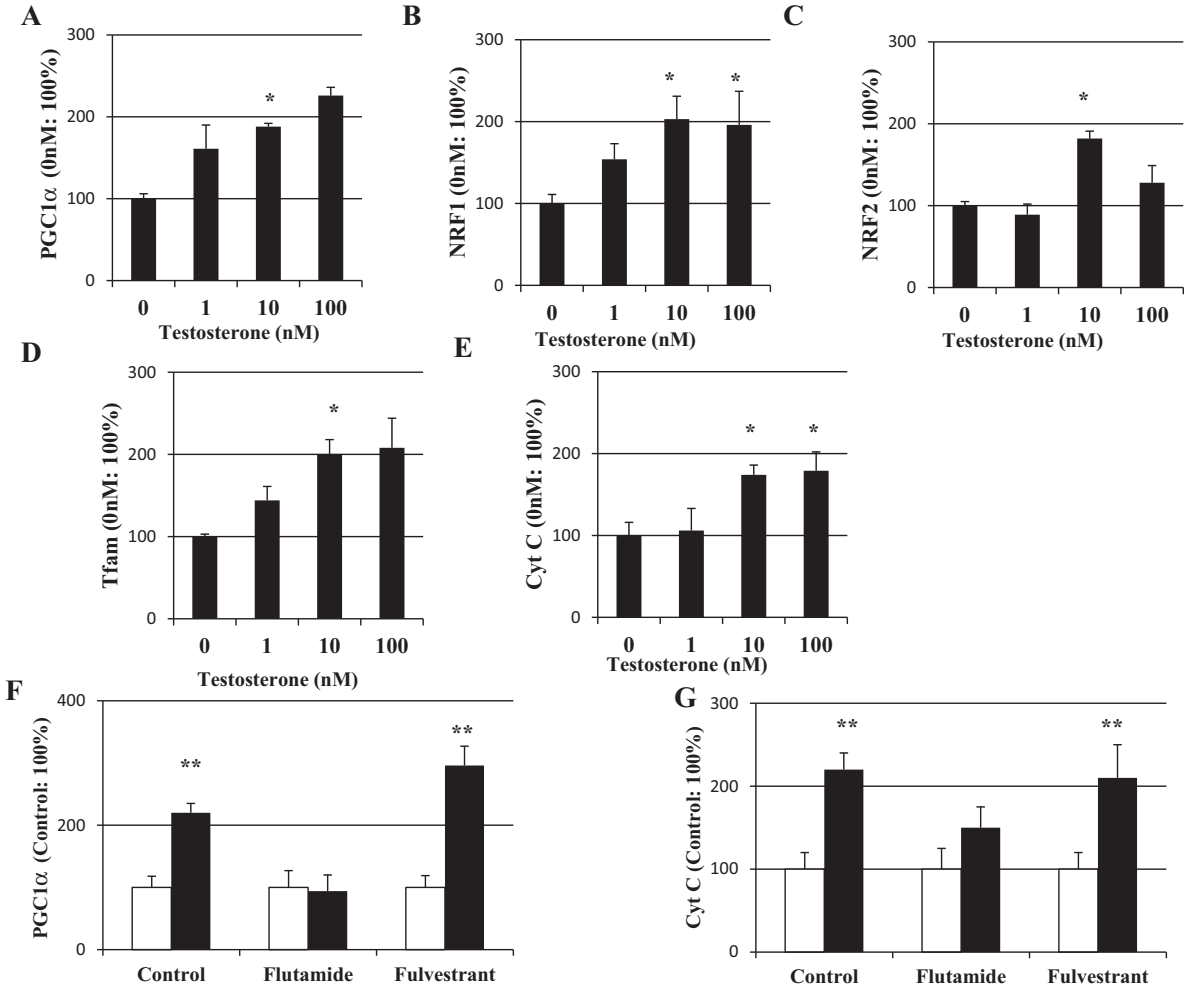

Fig. 4. Effect of treatment with testosterone on expression of mRNA related to mitochondrial biogenesis in C_2C_{12} myotubes. (A–E) Fully differentiated C_2C_{12} myotubes were incubated with various concentrations of testosterone for 48 h, and expression levels of PGC1α (A), NRF1 (B), NRF2 (C), Tfam (D) and cytochrome *C* (E) mRNA were measured ($n = 6$, *: $P < 0.05$, **: $P < 0.01$, vs testosterone 0). (F, G) Fully differentiated C_2C_{12} myotubes were incubated with (black) or without (white) 10 nM testosterone, and 1 μM flutamide, or 1 μM fulvestrant for 48 h. The expression levels of PGC1α (F) and cytochrome *C* (G) mRNA were shown.

activity and androgen signal may not be correlated linearly. Although we are unable to explain the underlying mechanism, we speculate that it might be due to some testosterone-induced action on the central nervous systems. In any case, the increase in energy expenditure and resultant weight loss despite reduced locomotor activity in testosterone-treated mice is derived from an increased basal metabolic rate.

Next we examined mitochondrial biogenesis. PGC1α was identified as a stimulator of mitochondrial biogenesis in muscle cells [20]. Recently, PGC1α is known to regulate mitochondria biogenesis in a tissue-specific manner [10]. The activity of PGC1α is regulated on both the transcriptional level, such as PPAR family, cAMP response element, mTOR, and post-transcriptional level, including Akt, AMPK and Sirt1 [21]. Mitochondrial biogenesis requires very complex processes. Expression of mitochondrial proteins encoded in the nucleus is regulated by NRF1 and NFR2. Additionally, proteins encoded in the mitDNA are under the control of Tfam, the expression of which is regulated by NFR1 and NFR2 [22,23]. Our data demonstrate clearly that treatment with testosterone results in up-regulation of PGC1α and subsequent mitochondrial biogenesis in skeletal muscle. Moreover, we observed reduced expression of PGC1α and mitochondrial biogenesis in skeletal muscle isolated from ARKO. Although treatment with testosterone increased the serum testosterone level up to more than 10-fold in vivo, testosterone at physiological concentrations is sufficient to increase mitochondrial biogenesis in C_2C_{12} myotubes. On the other hand, ARKO exhibit the opposite phenotype to mice treated with testosterone. As shown in Fig. 1K, the circulating testosterone level is suppressed in ARKO due to atrophic testis [24]. Since low testosterone concentration and blockade of AR lead to decreased mitochondrial biogenesis, as shown in Fig. 4F and G, we speculate that the serum testosterone level and expression level of AR may exert an equal influence on mitochondria biogenesis in muscle in ARKO. As the estrogen receptor antagonist, fulvestrant, failed to influence the testosterone-induced mitochondrial biogenesis, these effects of testosterone must be mediated via the androgen receptor.

We indicate that testosterone-induced increased mitochondrial biogenesis in skeletal muscle accounts for the testosterone-induced increase in energy expenditure. Contrary to our expectation, the mitochondrial biogenesis was not increased with treatment with testosterone in BAT. As the expression of PCG1α is positively regulated by activation of PPARγ in adipocytes [23], testosterone-induced reduction of PPARγ [5] might attenuate the increase of PGC1α in WAT and BAT. Actually, the effect of androgen on BAT has been controversial. Treatment with testosterone suppressed the expression of uncoupling protein 1 (UCP-1) [25] in cultured BAT, whereas UCP-1 expression was severely reduced in ARKO [17].

Our results indicate that skeletal muscle is a major organ contributing to androgen-induced weight loss. Myocyte-specific ARKO are reported to display lower body weight and visceral fat weight than their wild type littermates [26]. Conversely, selective overexpression of AR in myocytes of wild type rats and in rats carrying the testicular feminization mutation (a loss of functional AR in the whole body) heve been reported to result in reduced adiposity, increased O_2 consumption and increased mitochondrial volume in muscle [27,28]. Fernando et al. concluded that increased AR signaling in myocytes is sufficient to decrease fat volume [27]. These results are consistent with those of our study. However, their results indicated that AR signaling increased the amount of fast twitch myofibers [28], which was not in agreement with our findings. Further investigation of the molecular interaction between AR and PGC1α will be needed to clarify these discrepancies.

In conclusion, our results demonstrated that testosterone-induced reduced adiposity is associated with increased mitochondrial biogenesis in skeletal muscle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.051.

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